Progress in the Reaction of Pyridine Nucleotide-Dependent Enzymes. Part I

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REVIEW

Progress in the Reaction of Pyridine Nucleotide-Dependent Enzymes. Part I

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1. REDUCTION OF CARBONYL COMPOUNDS

1.1 Alcohol Dehydrogenase

1.1.1 Introduction

NAD(P)$^+$-dependent alcohol dehydrogenases [ADH; EC 1.1.1.1–2][1,2] catalyze the interconversion of various alcohols and corresponding aldehydes or ketones:

$$RR'CHOH+NAD(P)^+ \rightleftharpoons RR'CO+NAD(P)H+H^+ \quad (1)$$

Of numerous ADH, two enzymes from horse liver and from yeast have received much attention, and the scrutiny of the former enzyme in particular has greatly contributed to the understanding of the reaction mechanism of ADH.

The horse liver enzyme (LADH) is a dimer (Fig. 1) of molecular weight of 80,000 comprised of at least two distinctive subunit types, E and S ("ethanol-active" and "steroid-active", respectively).[3–6] Most of the studies on LADH have been focused on EE isoenzyme, with either purified[7–9] or EE predominant preparations, whereas some paralleling studies have been performed on SS isoenzyme.[9–12] Their primary structures are different in the position of six amino acids,[13,14] which seems to explain the difference in their catalytic properties. It has also been pointed out that the replacement of only one of six amino acids by another one results in the appearance of "steroid-activity".[15] ADH is ordinarily a zinc-metalloenzyme, and LADH contains four zinc atoms per dimer which have been classified into catalytic and non-catalytic pairs. The catalytic zinc can be specifically depleted with concomitant loss of enzymic activity or substituted by other metals with distinctly reconstituted activities.[16–20] The enzyme is strongly inhibited by metal chelating agents such as pyrazole,[21–23] and the fact has been well exploited by mechanistic studies.[24,25] On the other hand, yeast cytoplasmic enyzmes (YADH) are tetramers of molecular weight of about 145,000 and ecah subunit contains only "one" catalytic zinc atom.[1,26] In spite of these differences, the fundamental structures of YADH subunits have been

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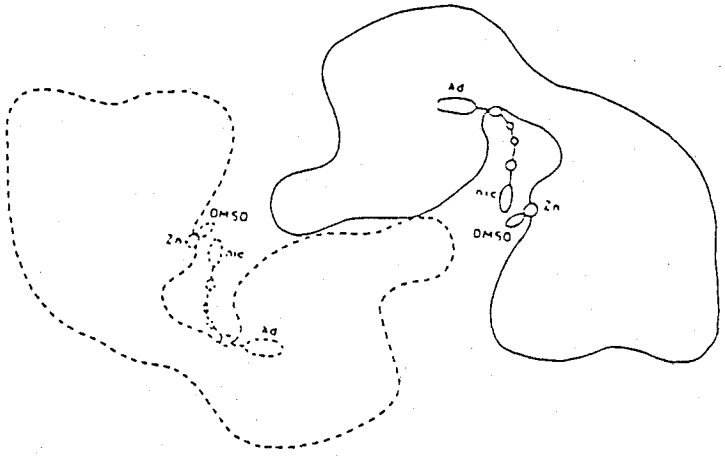


Fig. 1. Schematic drawing of the ADH-NAD+-DMSO complex with the active site zinc ion.

considered to be very similar to that of LADH, based on the comparisons of their amino acid seuqences (Fig. 2).[27,28] Although physiological roles of mamalian enzymes are still not clear, "steroid-activity" may be of intrinsic importance[29,30] rather than the activity toward ethanol, because the latter is not a normal bodily constituent.[31] Yeast enzymes can be said[32] to participate in either fermentation (Type I)[33] or ethanol oxidation (Type II).[34]

1.1.2 Molecular Structure and Substrate Binding

Extensive X-ray crystallographic studies have been made on LADH, including apoenzyme,[35–37] apoenzyme-coenzyme (or its analog) binary complexes,[38–40] complexes with various inhibitors,[22,23,41] modified enzymes,[42,43,16] and a variety of ternary complexes.[44–48]

As shown in Fig. 1, each subunit of LADH is divided into two domains,[36] *i.e.*, the coenzyme binding domain and the catalytic domain. These domains are separated by a deep active site cleft. The coenzyme binding domain is composed of residues 176–318 (See Fig. 2), and has the folding structure of essentially the same feature as found in many other NAD+-dependent dehydrogenases.[49] The catalytic domain is organized by residues 1–175 and 319–374. Two zinc atoms of the subunit bind to this domain. The catalytic zinc atom, which was firstly identified from the affinity to an inhibitor, 1,10-phenanthroline, and has been repeatedly confirmed by the affinity to several substrates and its analogs, is situated at the bottom of a deep pocket between the two domains, constructing a hydrophilic center in a strongly hydrophobic environment. The zinc atom is ligated by two sulfur atoms from Cys-46 and Cys-174 and one nitrogen atom from His-67. Selective modifications of Cys-46[50–54,42] or Cys-174[55,56] lead to a significant loss of the enzymic activity. The forth ligand in the apoenzyme is water or a hydroxide ion, depending on the pH. The environment of the active site zinc was discussed in comparison with those in other zinc-metalloenzymes.[57] In contrast to the catalytic zinc, the structural zinc atom is inert for substrate bindnig, because it is firmly ligated by four sulfur atoms

```
          10          20          30          40          50          60
STAGKVIKCKAAVLWEEKKPFSIEEVEVAPPKAHEVRIKMVATGICRSDDHVVSGTL V TPL
   SIPETQKGVIFYESHGKLEYKDIPVPKPKANELLINVKYSGVCHTDLHAWHGDWPLPTKL

         70          80          90          100         110         120
PVIAGHEAAGIVESIGEGVTTVRPGDKVIPLFTP QCGKCRVCKHPEGNFCLKNDLSMPRGTM
PLVGGHEGAGVVVGMGENVKGWRIGDYAGIKWLNGSCMACEYCELGNESNCPHADLSG

     130         140         150         160         170         180
QDGTSRFTCRGKPIHHFLGTSTFSQYTVVDEISVAKIDAASPLEKVCLIGCGFSTGYGSAVKV
                YTHDGSFQQYATADAVQAAHLPQGTDLAEVAPVLCAGITVY KALKS

   190         200         210         220         230         240
AKVTQGSTCAVFGL GGVGLSVIMGCKAAGAARIIGVDINKDKFAKAKEVGATECVNPQDYKK
ANLMAGHWVAISGAAGGLGSLAVQYAKAMGY RVLGIDGGEGKEELFRSIGGEVFIDFTKEKD

 250         260         270         280         290         300         310
PIQEVLTEMSNGGVDFSFEVIGRLDTMVTALSCCQEAYGVXVIVGVPPDSQNLSMNPMLLLSG
IVGAVLK ATNGGAHGVINVSVVEAAIEASTRYVR ANGTTVLVGMPAGAKCCSDVFNQVVK

         320         330         340         350         360         370
RTWRGAIFGGFKSKDSVPKLVADFMAKKFALDPLITHVLPFEKINEGFDLLRSGE SIRTILT
   SISIVGSYVGNRADTREALDFFAR  GLIKSPIKVVGLSTLPEIYERMEKGQVVGRYVVD


F       Horse Liver
TSK     Yeast
```

Fig. 2. Amino acid sequences for horse liver and yeast ADH's. A; Ala, C; Cys, D; Asp, E; Glu, F; Phe, G; Gly, H; His, I; Ile, K; Lys, L; Leu, M; Met, N; Asn, P; Pro, Q; Gln, R; Arg, S; Ser, T; Thr, V; Val, W; Trp, Y; Tyr, X; unidentified.

from Cys-97, Cys-100, Cys-103, and Cys-111.

The catalytic domain in dimeric LADH undergo distinct conformational changes upon the formation of a ternary complex, whereas the other parts of the enzyme including the coenzyme binding domains, active site clefts, and the main subunit interaction area remain virtually unchanged in their conformations. The total change is described as a rotation of the catalytic domains with respect to the central core. The front and rear parts move about 6–7 Å. The rotation, the pith of which consists in the movement of several loop residues such as 53–57 and 295–298, in combination with coenzyme and substrate bindings, makes the active sites shielded from the solvent. The nicotinamide-ring of the coenzyme is positioned close to the active site zinc. In an inactive ternary complex with NADH and DMSO, the inhibitor dimethylsulfoxide was found to be the fourth ligand of the catalytic zinc directly bound through its oxygen and displacing the water molecule in the apeoonzyme (Fig. 1).[47,48]

There have been much debate on the model of substrate binding, particularly in relation to the role of the catalytic zinc. Some mechanisms that assume an intermediate pentacoordinate zinc[57–60] as well as those with the possible formation of an outer sphere complex[61,62] instead of inner sphere ligation have caused vigorous

discussions. Recent crystallographic studies on several ternary complexes, however, strongly support the direct binding of the substrate to the catalytic zinc in tetracoordinate fashion.

The X-ray diffraction studies on crystals from an equilibrium mixture containing predominantly NAD^+ and *p*-bromobenzyl alcohol clearly demonstrated the direct coordination of the true substrate to the catalytic zinc during the normal reaction with LADH. These crystals have been reported to be catalytically active,[63] indicating that the ternary complexes investigated are productive ones. Further evidences that are consistent with the fact are as follows: 1) *trans*-4(N,N-dimethylamino)cinnamaldehyde (DACA) is known to react with LADH-NADH complex to form a transient intermediate with a characteristic absorption spectrum during the course of the reduction of this substrate.[64,65] The spectrum is very similar to those of model complexes between DACA and Lewis acids such as $Zn(NO_3)_2$.[66] The complex of LADH with 1,4,5,6-tetrahydronicotinamide adenine nucleotide (H_2NADH) and DACA,[67] which also exerts an absorption spectrum analogous to that of the transient intermediate described above, has been revealed by the X-ray analysis[48] to have a zinc-oxygen bond between DACA and the catalytic zinc. 2) An inhibitor, trifluoroethanol, which seems to be an inhibitor only because of the strong binding to the enzyme with biased equiliblium against the formation of an aldehyde (both owing to the electron-withdrawing substituent), has also been found to ligate directly on the catalytic zinc in good agreement with a productive binding mode.[45] 3) The analysis of resonance-enhanced Raman spectra[68,69] of an intermediate ternary complex of LADH with NAD^+ and *p*-(dimethylamino)benzaldehyde (DABA) also supports a direct binding mode to the aldehydic substrate.

Now, a schematic drawing for the productive substrate binding proposed by Brändén and Eklund[2] seems to be reliable. The zinc-bound oxygen of an alcohol can make a hydrogen bond with the hydroxyl group of Ser-48 to release a proton into the solvent through a relay system composed of the 2'-hydroxyl group of NAD^+-ribose and the imidazolyl group of His-51.[44] The Ser-48 is an almost exclusive candidate responsible for proton transfer from (or to) the substrate during the enzymic oxidoreduction by LADH.

1.1.3 Mechanism for Catalysis

Starting from the pioneering works by Theorell and his co-workers,[70,71] pKa values for various processes relevant to ADH action have been evaluated by extensive studies as summarized in Table 1. These values can be classified into four groups as indicated in Table 1. pKa's for the first three groups have usually been attributed to the zinc-bound water molecules in the apoenzyme, the enzyme-NAD^+ binary complex, and the enzyme-NADH binary complex, respectively. The abolishment of the pH-dependency for the binding of NAD^+ to an imidazole-bound enzyme[70] which has no zinc-bound water[41] strongly support the attribution. Since similar pKa values have been obtained for a water molecule on the zinc atom in several model zinc complexes,[90] there is no chemical problem for the values themselves in the rather large deviation from the value of 15.7 for free water molecule. Furthermore, the pKa differences between these three groups are reasonable from the viewpoint of the

Table 1. Optimum pKa Value for ADH Reactions

Group	pKa	Observation	Ref.
I	9.0–9.6	Rate for binding of NAD^+	72
	9.5	Rates for binding of NAD^+ and NADH	73
	9.2	Rates for binding of NAD^+ and NADH	74
	9.2	Rate for binding of anion	75
	9.2	Rate for binding of anion	76
	9.2	Rate for binding of pyrazole	77
	8.1	Rate for binding of *o*-phenanthroline	73
	9.2	Rate for binding of *o*-phenanthroline (reevaluation)	78
	9.2	Rate for binding of 2,2′-bipyridine	78
	9.8	Quenching of protein fluorescence with apoenzyme	79
	10.4	Rates for bindings of NAD^+ and NADH Distinct from $Zn(OH)_2$	80
II	18.0	Rate for E-NAD^+ dissociation	72
	8.0	Rate for E-NAD^+ dissociation	73
	7.6	Rate for E-NAD^+ dissociation (reevaluation)	74
	7.6	ΔH for binding of NAD^+	81
	7.6	Proton release with NAD^+ binding	82
	7.6	Rate for binding of benzyl alcohol to E-NAD^+	83
	7.6	Rate for binding 4f trifluoroethanol to E-NAD^+	84
	7.6	Rate for binding of pyrazol to NAD^+	77
	7.6	Quenching of protein fluorescence with E-NAD^+	79
III	11.2	Rate for binding of DACA to E-NADH	80
	>8.6	Rate bin&ing of NADH	81
	>9.9	Rate for reduction of β-naphthaldehyde	85
	>10	Rate for binding of anion to NADH	76
	>9.5	Rate for NADH dissociation from E-NADH	73
IV	6.4	Rate for oxidation of ethanol	86
	6.6–7.6	Rate for oxidation of benzyl alcohol	85
	6.4	Rate for oxidation of benzyl alcohol	87
	5.4	Rate for oxidation of $ClCH_2CH_2OH$	84
	4.5	Rate for oxidation of Cl_2CHCH_2OH	84
	8.3	Rate for oxidation of CF_3CH_2OH	88
	8.4	Rate for oxidation of benzyl alcohol with a modified enzyme	57
	6.4	Rate for dissociation of benzyl alcohol from E-NAD^+-ROH	83
	6.6	Rate for dissoication of naphtyl alcohol from E-NAD^+-ROH	83
	<5.5	Proton release with binding of CF_3CH_2OH to E-NAD^+	82
V	8.25	Rate for oxidation of ebnzyl alcohol by YADH	89
	8.25	Rate for reduction of acetaldehyde by YADH	89

electrostatic interaction between nicotinamide-ring and the zinc-bound hydroxide ion in an enzyme-NAD^+ complex and the oil-charge repulsion in an enzyme-NADH complex. As for the pKa for the quenching of protein fluorescence,[79] there has been an argument[91–93] against the attribution of the values to zinc-bound water, and the ionization of a tyrosine residue was suggested. The problem is, however, still controversial.[94] Further studies might be awaited to clarify the discrepancy.

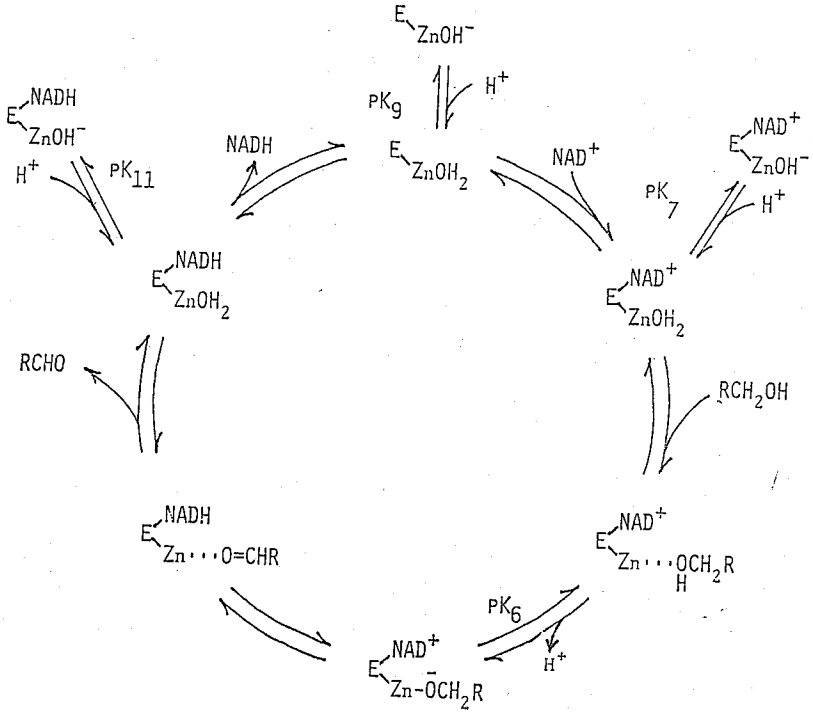
The values in the fourth group in Table 1, which control the rate of alcohol desorption from enzyme-NAD^+-alcohol complex and the conversion of ternary complex toward the oxidation of the alcohol, are attributable to the zinc-bound alcohols.

According to Kvassmann, Pettersson and their co-workers, the pKa value for an enzyme-NAD+-alcohol complex is linearly dependent on the pKa of the corresponding free alcohol with Brønsted α value of about 0.6, which sustains the attribution.

These data can be accounted for in a unified fashion by employing a scheme proposed by Kvassmann and Pettersson (Scheme 1),[82] which is a revised presentation of a scheme that had been proposed[1] based on X-ray crystallographic data and the results from earlier studies.[71,82] The order of substrate binding is drawn in Scheme 1 in a sequenced manner following the classical studies[8,12,72,95–100] although there must be partial randomness to a varing extent depending on the substate employed.[100–105]

For the oxidation of an alcohol, the first step is the binding of NAD+, then conformational isomerization[106–108] takes place to bring about the pKa shift from about 9.2 (pK_9) to about 7.6 (pK_7). The rate of NAD+ binding depends on the protonation state of an enzyme functional group of pK_9 which has been considered as the active site zinc-bound water. The easiness of subsequent binding of an alcoholic substrate to the enzyme-NAD+ binary complex also prefers the protonated state of the complex. In this respect, the reaction scheme is decisively different from the earlier proposal. Ionization of the enzyme-NAD+ complex is considered as a side reaction as shown in Scheme 1.

The alcohol in the resulted enzyme-NAD+-alcohol complex is in a proton dissociation equiliblium at around the pKa of about 6 (pK_6) with its alcoholate form prior to the hydride-equivalent transfer step. The postulation of a separate proton



Scheme 1

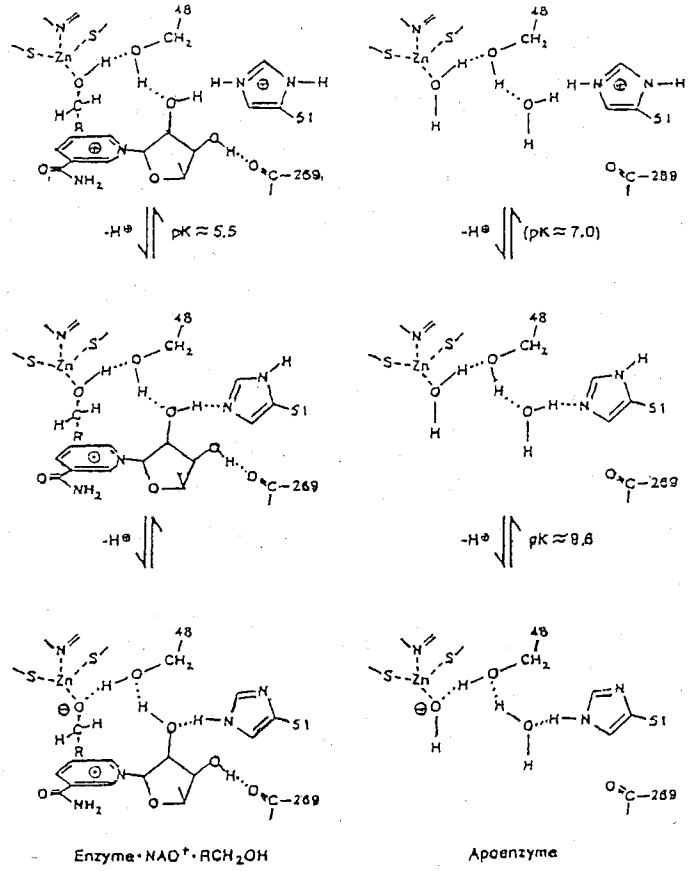
transfer step distinct from that of hydride-equivalent transfer is highly likely considering the substituent effects on pK_6. This regulates both desorption of alcohol and conversion of ternary complex. The independency of proton and hydride-equivalent transfer steps has also been substantiated by detailed inspection of solvent isotope effects on the LADH reaction.[25] Under a single turnover condition,[24] LADH-catalyzed reduction of aromatic aldehydes has been found to be biphasic. The rapid transient phase correpsonds to the process of ternary complex interconversion, *i.e.*, of hydride-equivalent transfer. The deuterium solvent isotope effect[25] for the rapid phase during the reduction of aromatic aldehydes was proved to be 1.0 ± 0.1, independent of pH. For the slow transient, on the other hand, inverse isotope effects of 2–3 were obtained at high pH's with a decreased value of 0.87 at pH 6.0. The rate of slow transient phase was determined by that of alcohol desorption from the ternary complex. These data are evidently consistent with the uncoupled transfer of proton from the hydride-equivalent transfer and rule out a concerted mechanism for hydrogen transfer previously proposed[109–111] to explain comparatively samll ρ values[109–113] obtained for LADH- and YADH-catalyzed reactions (Table 2). Solvent isotope effects in YADH catalyzed reaction also led Klinman and her co-workers to a conclusion to oppose any concerted mechanisms for hydrogen transfer.[114,115]

Further experimental results[116] that are inconsistent with a mechanism involving a general acid-base catalysis have been reported for the reduction of DACA (cf. 1.1.2) by LADH and NDAH. The rate of appearance of the characteristic absorption from an intermediate was pH-independent, whereas the rate for its disappearace depended on pH. That is, the rate of the process decreased largely with the increase in pH, and primary kinetic isotope effects (2.8 at pH 4.33) decreased to the abolition (1.0) above pH 7. The fact can be easily explained provided one assumes a proton transfer step subsequent to hydride-equivalent transfer in the process. Detailed analysis on primary kinetic isotope effects in YADH-catalyzed reaction at various pH also suports a stepwise mechanism.[117]

As for the molecular mechanism for proton transfer, a scheme based on the X-ray structure[44] together with supporting evidences from chemical modifications[118] has been proposed (Scheme 2). As already mentioned in 1.1.2, the proton transfer system involving His-51, 2'-hydroxyl group of NAD^+-ribose, and Ser-48 has been

Table 2. Summary of ρ value for ADH reactions

Reaction	ρ	Ref.
Oxidation of *p*-substituted benzyl alcohol by LADH	$\rho = -0.76$	109
Oxidation of 2-substituted ethanol	$\rho^* = -1.8$	110
Oxidation of *p*-substituted benzyl alcohol	$\rho^+ = -0.2$	57
Reduction of *p*-substituted benzaldehyde	$\rho^+ = +1.1$	57
Reduction of *p*-substituted benzaldehyde	$\rho = +2.2$	111
by YADH	$\rho^+ = +2.1$	112
Association Constant with *p*-substituted	$\rho = -0.85$	111
benzaldehyde	$\rho^+ = -0.92$	112
Equiliblium conversion of *p*-substituted benzaldehyde to the corresponding alcohol	$\rho = +1.5$	111



Scheme 2

proposed. Carefully designed chemical modifications of LADH such as the treatment with diethyl pyrocarbonate after acetimidylation of all accessible lysin residues revealed the existence of at least one essential histidine residue, probably His-51. From the pH-dependency of the inactivation of apoenzyme, an unusually high and significant pKa value of 9.6 was obtained, and the value was interpreted as identical to pK_9 of the zinc-bound water in the proton relay system. The possible involvement of plural protons in the proton relay system in LADH was also suggested by proton inventory experiments.[119]

The next step in the LADH reaction is the conversion of ternary complex by hydride-equivalent transfer from the zinc-alcoholate to NAD^+ (See 1.1.4), followed by the desorption of the resulted aldehyde. In the next and the last setp, dissociation of NADH, which is usually the rate-determining step in LADH reaction, completes the forward reaction.

The pH-independency of several processes in LADH action within a usual pH range including the NADH dissociation,[73] the rapid transient phase in aldehyde reduction,[85] etc. is explainable without any difficulties as a result of pKa perturbation of zinc-bound water to a rather high value of 11.2 (pK_{11}) in enzyme-NADH

complex.

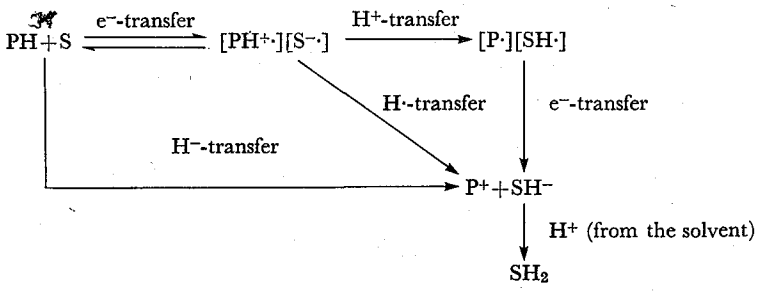
The asymmetric pH-dependency in LADH-catalyzed oxido-reduction is probably originated from the difference between zinc-bound alochol-alcoholate equiliblium (pK_6) in enzyme-NAD^+-alcohol complex and zinc-bound water-hydroxide equilibrium (pK_{11}) in enzyme-NADH complex. Contrary to LADH-catalyzed oxidoreduction, apparent symmetry of pH-dependency in YADH-catalyzed reaction (the group V in Table 1) has been reported,[89] which is compatible with the existence of a single functional group to regulate both oxidation and reduction. More detilaed studies with varing substrates are, however, required to corroborate the hypothesis. The apparent symmetry might be accidental coincidence between "pK_6" and "pK_{11}", which seems not so surprising considering the metabolic role of YADH (Type I). YADH solely engages in the reduction of acetaldehyde as a result of the adaptative evolution. For the purpose, a high "pK_6" value seems much advantageous.

After all, the chemical nature of ADH reaction might be safely explained as an electrophilic catalysis by the Lewis acid,[113] the active site zinc, through the formation of zinc-alcoholate bond, which reminds us of essential similatities of the enzymic reaction to Meerwein-Pondorff-Verley reduction and its reverse Oppenauer oxidation.

1.1.4 Mechanism of Hydride Transfer

The mechanism for (net) hydride transfer from and to dihydronicotinamides including NAD^+-NADH, $NADP^+$-NADPH, and their analogs in enzymic and mimetic reaction systems has been in continuing controversy. Since the pioneering works by Westheimer and his co-workers,[120,121] a direct transfer of a hydride in "one-step" had been believed widely over a couple of decades. Later, in 1971, Steffens and Chipman questioned the mechanism based on their results from kinetics.[122] Re-investigation of Westheimer's reaction system by means of ESR spectroscopy as well as detailed anlayses on the reaction products[123,124] also supported the Stefens and Chipman's proposal that the reduction is composed of multi-steps and the transfer of one electron from the dihydronicotinamide-ring to a substrate initiates the total reduction. Since then the mechanism of this (net) hydride transfer reaction has been a subject of large discussion.[125–129]

The (net) hydride transfer may be explained by three different processes shown in Eq. 2, where PH and S represent a dihydropyridine derivative and a substrate, respectively.



(2)

The mechanism of reduction has been investigated extensively with mimetic systems. Since detailed discussion on this topic will appear in elsewhere, we will

focus the topic on enzymic systems and evidence obtained so far will be described only briefly.

Already in 1957, it was reported that ternary complexes including ethanol-NAD^+-ADH, acetaldehyde-NADH-ADH, and other biological systems exert ESR signals that may indicate the presence of charge-transfer complexes as intermediates of the enzymic reactions.[133] However, the signals were so complicated that they could not be attributed to any particular species.

Substituent effects as well as large kinetic isotope effects associated with the reductions of substituted and unsubstituted benzaldehydes or with the oxidations of substituted and unsubstituted benzyl alcohols with a YADH system, on the other hand, led Klinman to conclude that the reaction does not involve the intermediate.[111,112,114] Few years later, however, Klinman and her co-workers studied α-secondary kinetic isotope effect of the same reduction and concluded that the reaction passes through a radical intermediate.[115]

In the reduction of pyruvate to lactate, isoenzymes of lactate dehydrogenase from pig heart and pig muscle exerted no kinetic deuterium isotope effect.[134] The results reveal that the process involving the movement of hydrogen nucleus does not constitute the rate-determining step. Here, the isomerization of substrate-NADH enzyme ternary complex to an "active complex" is suggested to be the rate-determining step. A question whether the "active complex" corresponds to a "charge-transfer complex" or to a "conformationally distorted complex" remains unsolved.

Theoretical considerations on kinetic isotope effects for the reduction with ADH suggests that there is little charge on the carbonyl carbon of the substrate.[135] The out-of-plane bending of C_4-H in the dihydropyridine-ring and tunneling effect contribute largely to the large kinetic isotope effect.[135–137]

Thus, scattered results prevent us from obtaining an unequivocal conclusion on the mechanism of enzymic (net) hydride transfer process.

In addition to pysical organic techniques mentioned above, product analyses have been done from the viewpoint to diagnose the reaction mechanism. Namely, the reaction with LADH was investigated by means of several chemically based radical probes such as nortricyclanone, 2,2-dimethyl-5-hexenal, and *cis*-3-phenylpropenal.[138] These substrates did not afford the rearranged products indicating that there is no radical intermediacy in the reaction. Similar result was obtained in LADH oxidation of α-hydroxyalkylcyclopropanes.[139] It is known that cyclopropylmethyl free radical isomerizes into butenyl free radical quantitatively,[140] but the whole product from the enzymic reduction reserved the cyclopropyl moiety.

Despite the results that support the one-step mechanism, the multi-step mechanism cannot be discarded at present, because the entropically frozen system such as a reaction in an active site of enzyme is easily expected to afford the unisomerized product. It is not surprising in organic reaction system that an in-cage reaction results in the product unaffected by enveronmental conditions.

Thus, both for enzymic and mimetic reactions, discussions on the mechanism of (net) hydride transfer will be continued further. In this review, we will denote the transferring species as a "hydride-equivalent".

1.2 Lactate Dehydrogenase

1.2.1 Introduction

NAD+-dependent L-lactate dehydrogenase [LDH; EC 1.1.1.27][141] catalyzes the reversible oxidation of L-lactate to pyruvate:

$$CH_3CH(OH)COOH + NAD^+ \rightleftharpoons CH_3COCOOH + NADH + H^+ \quad (3)$$

LDH from various sources are usually tetramers of molecular weight of about 140,000. In higher animals, there are A (M; muscle type), B (H; heart muscle type), and C (X; testis type) subunits. It is known that these subunits are encoded by separate genes (Fig. 3),[142–144] and isoenzymes composed of these subunits are distinguishable each other by electrophoresis,[115,146] immunochemical methods,[147–151] chemical composition,[142–154] and kinetic properties.[154,155] Affinity chromatography and ion exchange chromatography are useful for isolation of each isoenzyme in practical scale.[150,156–159] In somatic tissues, LDH exists as isoenzymes composed of nonrandom combinations[160] of A and B subunits to form tissue-specific isoenzyme pattern, although random (statistical) combinations have been demonstrated *in vitro*.[161] A time lag in the protein synthesis for each subunit might be an important factor for the results. The A/B ratio differs from tissue to tissue.[150,162–164] It also differs, as a result of variable expresison of the genes,[162] in the same tissue of an organ at different developmental stages.[165] It has been recognized that comparatively anaerobic tissues such as skeletal muscle and liver prefer B type which is more adequate for pyruvate reduction than A type, and *vice versa*.[166] The C type of LDH has been found uniquely in mature testes and spermatozoa.[156,167] and is known to localize in the mitochondria.[167] In mouse, rabbit, human, ram, and dog, only the C_4 isoenzyme has been detected, whereas multiple forms of LDH C have been reported in rat, bull, pig, pigeon, etc.[156] LDH C is more sensitive to pyruvate inhibition than the lactate inhibition. Thus, LDH C might play an important role for the oxidation of lactate *in vivo*. Although the physiological role of the C type LDH is still obscure, one proposal has been that the C_4 isoenzyme participates in a transmitochondrial oxidation-reduction shuttle.[167] These animal enzymes do not exhibit cooperativity in the coenzyme binding, whereas some allosteric activation by anions such as chloride[168] and phosphate[169] has been reported. Usually LDH from bacterial sources are allosterically activated by a large "anion", fructose-1,6-bisphosphate,[170–177] and these in particular are called conventionally as allosteric LDH's. The complete sequence of an allosteric LDH from *L. casei* has been determined and a considerable homology of about 37% (and of 70% within the active site) to verteblate LDH has been documented.[172] LDH is not a metalloenzyme in contrast to ADH.[151] It is suspected that LDH does not require the metal ion-catalysis because the substrate for this enzyme is much more reactive toward the reaction than those for ADH.

1.2.2 Molecular Structure and Substrate Binding

Crystallographic studies of LDH include those on apoenzyme (dogfish muscle A_4,[178] pig heart B_4,[179] and mouse C_4[180]), binary complexes (dogfish A_4[181–184]), abortive ternary complexes (dogfish A_4,[185,186] pig A_4,[187] and pig B_4[188]), and an active ternary complex (pig B_4[189]).

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         10         20         30         40         50         60
ATLKDKLIGHLATSQEPRSYNKITVVGVGAVGMACAISILMKDLADEVALVDVMEDKLKGEMM
  LKDHLIHNVHKEEHAHAHNKXXXXXXXXXXMACAISILMKBLABZLTLVDVVZBKLKGEMM
ATLKDQLIHNLLKEEH VPHNKITVVGVGAVGMACAISILMKELADEIALVDVMEDKLKGEMM
ATLKEKLITPVAAGST VPSNKXXXXXXXXXXMACAISILGKGLCDELALVDVLEDKLKGEMM
ATLKEKLIAPVAQQETTIPNNKITVVGVGQVGMACAISILGKSLTDEVALVDVLEDKLKGEMM
STVKEELIQNLVPDKL  SRCKITVVGVGDVGMACAISILLKGLADELALVDADTDKLRGEAL
STVKEELIQNLAPDKQ  SRCKITVVGVGDVGMACAISILLKGLADELALVDADEDKLKGEAL

      70         80         90        100        110        120
DLQHGSLFLHTAKIVSGKDYSVSAGSKLVVITAGARQQEGESRLNLVQRNVNIFKFIIPNIVK
DLQHGSLFLKTPKITSGKDYSVTAHSKLVIVTAGARQQEGESRLNLVQRNVNIFKFIIPNVVK
DLQHGSLFLRTPKIVSGKDYNVTANSRLVVITAGARQQEGESRLNLVQRNVNIFKFIIPNIVK
DLQHGSLFLQTHKIVABKBYAVTANSKIVVVTAGVRQQEGESRLNLVQRNVNVFKFIIPZIVX
DLQHGSLFLQTPKIVANKDYSVTANSKIVVVTAGVRQQEGESRLNLVQRNVNVFKFIIPQIVK
DLLHGSLFLSTPKIVFGKDYNVSANSKLVIITAGARMVSGQTRLDLLQRNVAIMKAIVPGVIQ
DLLHGSLFLSTPKIVFGKDYSVSANSKLVIITAGARMVSGQSRLALLQRNVTIMKAIVPGVIQ

  130        140        150        160        170        180
NSPDCIILVVSNPVDVLTYVAWKLSGLPMHRIIGSGCNLDSARFRYLMGERLGVHSCSCHGWV
YSPDCXXXXXXXXXXXXXVAWKISGFPKHRVIGSGCNLDSARFRHLMGERLGIHPLSCHGWI
YSPNCKLLVVSNPVDILTYVAWKLSGLPKHRVIGSGCNLDTARFRYLMAERLGIHPTSCHGWI
XXXXXXXXVVSNPVDILTYVTWKLSGLPKHRVIGSGCNLDTARFRYLMAERLGIHPTSCHGWI
YSPNCIIIVVSNPVDILTYVTWKLSGLPKHRVIGSGCNLDSARFRYLMAEKLGVHPSSCHGWI
NSPDCKIIVVTNPVDILTYVVWKISGFPVGRVIGSGCNLDSARFRYLIGEKLGVNPTSCHGWV
NSPDCKIMIVTNPVDILTYVVWKISGLPVSSVIGSGCNLDSARFRYLIGEKLGVNPTSCHGWV

190        200        210        220        230        240        250
IGEHGDSVPSVWSGMWNA    LKELHPELGTNKDKQDWKKLHKDVVDSAYEVIKLKGYTSWAI
VGQHGDSSVPVWXXXXXXXXXXXNLHPDMGTBABKZXXKEVHKQVVDSAYEVIKLLGYTSWAI
LGEHGDSSVPVWSGVNVAGVSLKNLHPELGTDADKEHWKAVHKEVVDSAYEVIKLKGYTSWAI
LGEHGDSSVAVWSGVNVAGVSLQQLNPAMGTBKBSENWKEVHKQVVESAYEVIRLKGYTNWAI
LGEHGDSSVAVWSGVNVAGVSLQQLNPEMGTDNDSENWKEVHKNVVESAYEVIKLKGYTNWAI
LGEHGDSSVPIWSGVNVAGVTLKSLNPAIGTDSNKQHWKNVHKQVVEGGYEVLDMKGYTSWAI
LGEHGDSSVPVWSGVNVAGVTLKSLNPAIGSDSMKQEWKTVHKQVVDGGYEVLDLKGYTSWAI

       260        270        280        290        300        310
GLSVADLAETIMKNLCRVHPVSTMVKDFYGIKDNVFLSLPCVLNDHGISNIVKMKLKPNEEQQ
GLSVADLAETIMKNLRRVHPISTAVKGMHGIKDDVFLSVPCVLGXXXXXXXXXILKPDEEEQ
GLSVADLAESIMKNLRRVHPISTMIKGLYGIKENVFLSVPCILGQNGISDVVKVTLTPEEEAH
GLSVAZLCZTMLKNLYRVHSVSTLVKGTYGIQDDVFLSLPCVLSASGLTSVINQKLKDDEVAK
GLSVADLIESMLKNLSRIHPVSTMVQGMYGIENEVFLSLPCVLNARGLTSVINQKLKDDEVAQ
GLSVTDLARSILKNLKRVHPVTTLVKGFMGIKEEVFLSIPCVLGESGITDFVKVNMTAEEEGL
GLSVTDLAESILKNLKRVHAVTTLVKGLYGIKEEIFLSIPCVLGESGITDLVKVNMNTEEEAL

    320        330
LQKSATTLWDIQKDLK F      Dogfish Muscle
IKKSATTLWGIQKELQ F      Chicken Muscle
LKKSADTLWGIQKELQ F      Pig     Muscle
LKKSADTLWSIQKDLKDL      Chicken Heart
LKNSADTLWGIQKDLKDL      Pig     Heart
LKKSADTLWNMQKNLE L      Mouse   Testis
FKKSCDILWNIQKNLE L      Rat     Testis
```

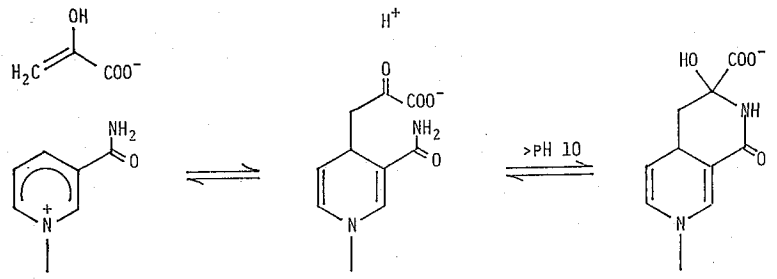
Fig. 3. Amino acid sequences for various LDH's.

Usually, each LDH subunit is divided into four domains: N-terminal arm, coenzyme binding domain, loop and helix αD region, and substrate binding (catalytic) domain. N-Terminal arm (residues 1–20) might engage in subunit interactions across the R-axis in LDH tetramer.[141] Bacterial LDH, however, lacks the first 14 residues of the N-terminal arm in vertebrate LDH and yet tetramer,[143,172,177] which makes the role of the N-terminal arum unclear. The coenzyme binding domain, the loop region, and the catalytic domain are composed of residues 22–97 and 115–164 (a conventional numbering based on the results from earlier sequencing works, which has been widely employed in various reports including those of X-ray

studies) (21–95 and 118–163; a numbering shown in Fig. 3, which hereafter is given in parentheses), 98–114 (96–117), and 165–331 (164–333), respectively. The coenzyme is placed in the bottom of a cleft with the nicotinamide-ring buried deeply within the subunit.[182] Upon the formation of ternary complex, definite conformational changes occur in regions around the active center.[185,186,189] Consequently, the loop covers the active site pocket and the C-terminal helix αH. At the same time, the unreactive B side of nicotinamide is set in hydrophobic environment composed of Thr-264 (248,) Ile-250 (252), Val-138 (136), and Leu-167 (165)[189] Arg-101 in the loop forms, changing its position by 13 Å, an ionic bond with the pyrophosphate moiety of the coenzyme. The movement of the loop not only introduces various charged groups into the vicinity of the substrate binding site, but also is responsible for conformational change of the essential His-195 which moves about 2 Å toward the substrate.

Solvent molecules in the active site seems to be excluded through a hydrophilic gap between the loop and the rigid part of the molecule, which is lined by Glu-107 (104), Arg-109 (106), Glu-194 (192), Asp-197 (195), Asp-231 (233), Asp-234 (236), Tyr-237 (239), Glu-238 (240), and so on.[179] Increase in the susceptibility to proteolysis of an immobilyzed LDH upon theformatoin of productive ternary complex supports the conformational changes.[190] ESR studies employing a spin-labeled active analog of NAD^+ have provided experimental data that are consistent with a fluctuation of the loop between open (up) and closed (down) forms in solution.[191] The rigidly closed loop is a trait of the ternary complex, and at the stage of the binary E-coenzyme complex, an open conformer is still predominant[182,191] at least in the case of dogfish A_4 and pig B_4 isoenzymes. In addition, the primary structure of the loop region of A and B subunits are almost completely conserved, whereas C subunits have a rather different (about 50% conservation between A or B and C subunits) sequence.[143] Curiously, the loop in the crystals of apo LDH C_4 has been found to have a closed ternary-like conformation. This may be the result of preferred crystallization from a fluctuating mixture of open and closed forms in solution, which may partly explain the low turnover number of the C_4 isoenzyme (5–10% of that of the somatic LDH).[180]

The structure of the productive ternary complex has been estimated by the analysis of various model complexes. An abortive E-NAD^+-pyruvate complex, which was firstly investigated in detail, was found to be a binary complex in reality because it contains a covalent bond between NAD^+ and pyruvate. The complex is reversibly formed by the reaction of E-NAD^+ with the enol form of pyruvate.[192–195] The nucleophilic attack of pyruvate enol occurs stereospecifically at the C_4 position from the A side of the nicotinamide-ring (Scheme 3). The significance of this observation upon the mechanism of normal oxidoreduction with LDH is obscure, because the normal reaction requires the keto form.[192,196] At the same time, both pyruvate and NAD^+ are in oxidized states. The same adduct formation can easily be accomplished by non-enzymic base catalysis without, of course, any stereospecificity. In the case of the non-enzymic reaction, further bond formation between the amide nitrogen and the pyruvate carbonyl carbon occurs to give a cyclized adduct. The cycliza-

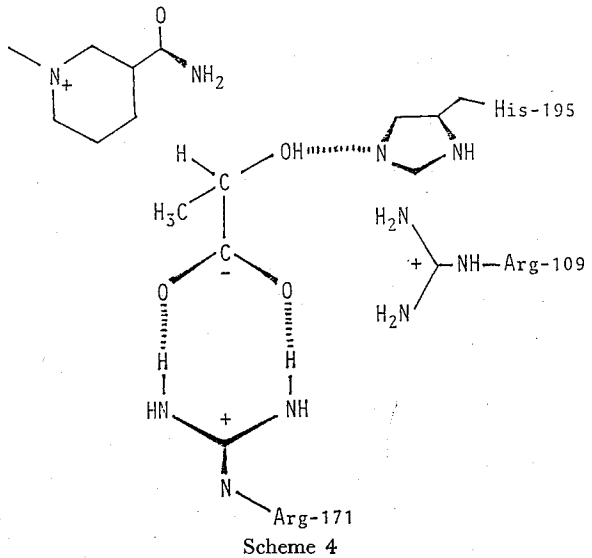


Scheme 3

tion reaction also occurs when the adduct formation is performed in the presence of LDH at a raised pH of 10, probably after the dissociation of the first adduct from the enzyme.[193,194] The possible physiolosical significance of the complex formation has been discussed.[141,197]

A most plausible mode of the productive substrate binding has been deduced. Scheme 4 shows a model built with the true substrate taking into account the elucidated structure of active site of an abortive complex as well as the result from chemical modification. The latter evidence indicates the presence of a single histidine[198] and several arginine[199] reidues essential for the catalytic activity.

The carboxyl group in the substrate forms a salt bridge with the guanidinium group of Arg-171. The imidazolyl group of His-195 is in contact with the alcoholic oxygen of the substrate through a hydrogen bond, thus acting not only as an acid-base catalyst but also as one of the "three points" in the stereospecific oxidoreduction of the substrate.[200] (The formation of an abortive complex may also be catalyzed by His-195.[193]) The substrate is placed between His-195 and the C_4 position of the nicotinamid-ring. Another arginine residue, Arg-109 shown in Scheme 4, one of the loop residues, is brought into the vicinity of the active site by the large move-



Scheme 4

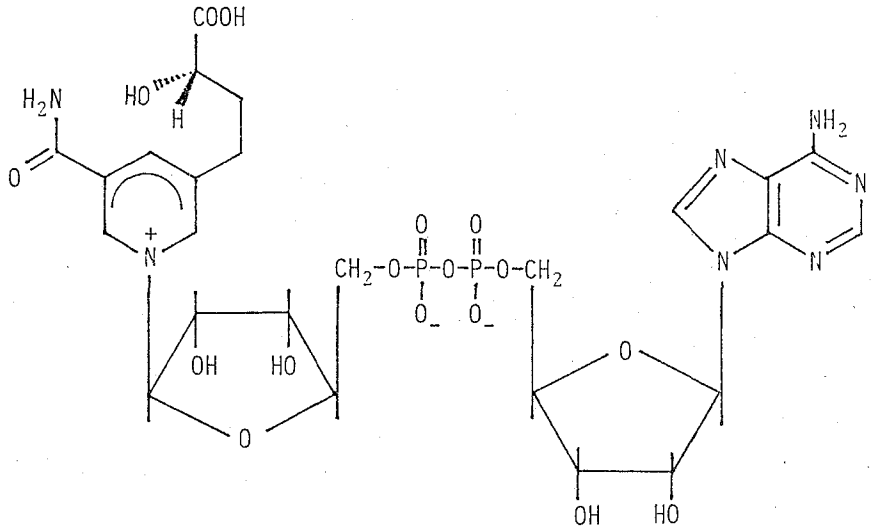
ment of about 23 Å.

The estimation has been substantiated by the analysis of an active "trenary" complex E-S-lacNAD+.[189] S-lacNAD+ has a structure in which (*S*)-lactate is covalently bound through a methylene spacer in the C_5 position of the nicotinamide-ring[201] and undergoes an intramolecular hydride-equivalent transfer in the presence of pig heart LDH to form 5-(2-oxalylethyl)NADH reversibly.[202] The elucidated mode of substrate binding was proved to resemble closely the earlier proposal.

Although considerable interest had been devoted on a possible participation of Cys-165 (163) in catalysis,[203–205] it is now not clear if an indispensable role is rendered to this cystein. X-ray data have indicated that the residue is at least not directly involved in LDH action. Furthermore, this cystein is out of the amino acid conservation with an alteration to threonine in the lobster tail[206,207] and *L. casei*[172] enzymes. These fcats, of course, do not necessarily exclude the "essentiality" of the residue(s). However, thiomethylation of Cys-165 in pig B_4 LDH[208–210] was found to retain full catalytic activity of the enzyme (*i.e.*, an equal V_{max} to that of the native enzyme), though the affinity of the enzyme for its substrates was markedly decreased (about 30 times weaker binding for pyruvate). Some additional steric hindrance carried into the active site by chemical modification of Cys-165 might prevent the movement of the essential His-195 toward the substrate binding site.[210] It is interesting to know if a nonpolar and small amino acid such as alanine can replace Cys-165 without abolition of the enzymic activity.

1.2.3 Mechanism for Catalysis

The reaction proceeds through an ordered pathway[211–216] with the initial addition of a substrate followed by the binding of coenzyme. LDH does not form an E-lactate nor an E-pyruvate complex in the absence of NAD+ or NADH. In the reaction with LDH, chemical processes are so rapid (essentially instantaneous reac-



S-lacNAD+

tions) that an equiliblium between ternary complexes (E-NAD^+-lac $\rightleftharpoons$ E-NADH-pyr) is achieved before the establishment of the steady state. As a result, k_H/k_D for overall reaction is 1.0.[134] The equiliblium constant changes depending on pH or concentration of lactate. There is a conservation of charge during the interconversion of the ternary complexes. An isomerization of the E-NADH-pyr complex leads to the dissociation of pyruvate, and further isomerization after the dissociation of pyruvate brings about a change of pKa of a group (probably His-195) in the enzyme to release a proton. The rate-determining step in the steady state of NADH formation is the step for the dissociation of NADH.

Concerning to the mechanism of the reaction with LDH, a modified scheme of "oil-water-histidine" mechanism by Parker and Holbrook[271] was presented recently by Grau and his coworkers.[189] The scheme takes a reversible domino effect into account in the reaction course. For the oxidation of lactate, NAD^+ binds to the enzyme initially to form an open loop binary complex predominantly, then, as already mentioned above, lactate binds to this binary complex to form a ternary complex, which results in the closure of the loop. In this complex, the nicotinamide moiety is forced into a hydrophobic environment, which may destabilize the positive charge on the N_1 position of nicotinamide. The oil-charge repulsion might lead to the electron flow to induce a positive charge on the C_4 position, which promotes the transfer of (net) hydride. A concomitantly developed charge on His-195 after proton transfer causes charge repulsion against Arg-109, which may lead to the dissociation of the Michaelis complex, pushing the loop to be opened.

For the reverse, introduction of Arg-109 into the active site of enzyme may lead to decrease in pKa of the protonated His-195 to facilitate the protonation of the carbonyl group in a substrate forming a (formal) carbocation. After the transfer of a hydride-equivalent, the oil-charge repulsion between the hydrophobic wall and the resulted positive charge on the N_1 position of nicotinamide leads to the break down of the complex.

1.3 Malate Dehydrogenase

1.3.1 Introduction

Malate dehydrogenase [MDH; EC 1.1.1.37][218] catalyzes the interconversion of L-malate and oxaloacetate.

$$\begin{array}{l} \text{HO-CHCOOH} \qquad\qquad \text{O=CCOOH} \\ \quad \text{CH}_2\text{COOH} + \text{NAD}^+ \rightleftharpoons \text{CH}_2\text{COOH} + \text{NADH} + \text{H}^+ \end{array} \qquad (4)$$

All eukaryotes have at least two distinct isoenzymes: mitochondrial (m-MDH) and cytoplasmic (soluble or supernatant) (s-MDH). The both enzymes are dimer [219,220] of molecular wight of about 70,000 comprised of two identical subunits.[221–226] Each subunit is catalytically active as a monomer.[227] The enzymes are synthesized in the cytoplasm and m-MDH is translocated into mitochondrial matrices guided by a signal peptide in the precursor,[228,229] whereas s-MDH remains in the cytosol. m-MDH is a key enzyme in the citric acid cycle,[230,231] and also constructs the malate-aspartate shuttle[230] in coopearation with s-MDH.

1.3.2 Molecular Structure and Mechanism for Catalysis

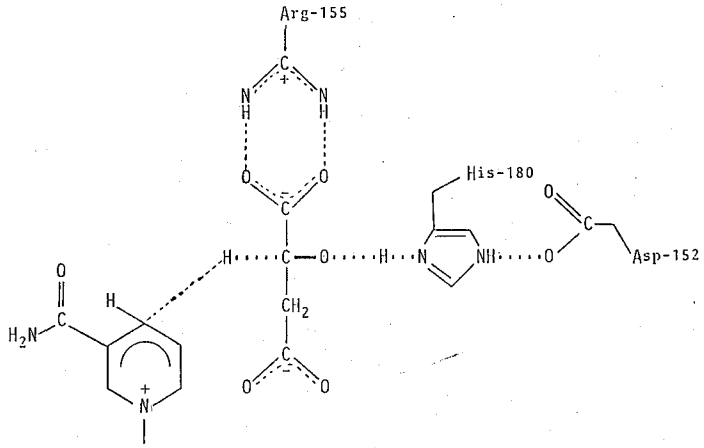


Fig. 4. Schematic representation of the active site in the MDH-NAD^+-malate complex.

As one of α-hydroxy acid dehydrogenases, MDH is similar to LDH in amino acid sequences,[232] X-ray structures,[233–236] essential amino acid residues ($His^{237-241}$ and $Arg^{242,243}$), and kinetic properties.[244,245] The formation of abortive ternary complex was observed with some MDH.[246] Lack of the N-terminal arm which exists in tetrameric LDH and/or the displacement of other amino acid probably makes MDH dimers. Other domain structures including the "loop" region are very similar to those in LDH. These facts may indicate essentially the same molecular mechanism for MDH reaction as estimated for LDH.

An estimated structuer of active site[236] for enzyme-NADH-oxaloacetate ternary complex is shown in Fig. 4. The 2-carbonyl oxygen interact with the protonated imidaoylzl group of an active site histidine by a hydrogen bond as in LDH. The 4-carboxyl group contacts with the solvent.

1.4 Isocitrate Dehydrogenase

1.4.1 Introduction

There is another group of dehydrogenases that catalyze the decarboxylative dehydrogenation of some β-hydroxy acids.[247] These include isocitrate dehydrogenase [ICDH; EC 1.1.1.41–42],[248–251] malic enzyme [ME; EC 1.1.1.38–40],[251–258] 6-phosphogluconate dehydrogenase [PGDH; EC 1.1.1.44,[259–261] etc.].

ICDH catalyzes the following reaction.

$$\begin{array}{l} H_2CCOO^- \\ HCCOO^- + NAD^+ \rightleftharpoons \\ HOCHCOO^- \end{array} \begin{array}{l} H_2CCOO^- \\ HCCOO^- + NADH + H^+ \\ O{=}CCOO^- \end{array}$$

oxalosuccinate

(a putative intermediate)

$$\rightleftharpoons \begin{array}{l} H_2CCOO^- \\ CH_2 + CO_2 + NADH \\ O{=}CCOO^- \end{array} \qquad (5)$$

2-oxoglutarate

NAD^+-dependent ICDH [d-ICDH; EC 1.1.1.41] and $NADP^+$-dependent ICDH [t-ICDH; EC 1.1.1.42] have rather different molecular properties each other. Pig heart enzymes have been the most studied ones. The mitochondrial d-ICDH is comprised of three dissimilar subunits (α, β, and γ) which occur in the ratio of 2 :1:1.[247] Each of them has a molecular weight of about 40,000[262–264] and exerts a catalytic activity to varing extents.[262] The enzyme is allosterically or "apparently" regulated by adenine nucleotides and citrate[265–267] as those from various sources.[268–271] The activators (Mn^{2+} and ADP) and the substrates (NAD^+ and isocitrate) bind to a half of the subunits (1 site per 2 subunits)[272] probably owing to strong negative cooperativity.[262,273] On the other hand, the pig heart t-ICDH is a dimer of molecular weight of about 115,000.[274,275] The enzyme has been found in both mitochondria and cytoplasm[276] and is a non-reguratory enzyme in contrast to d-ICDH.

ICDH requires a divalent metal cation.[277–283] The metal ion may act not only as a structural part of the substrate-metal ion complex but also as a Lewis acid catalyst for decarboxylation[281] as proposed for the non-enzymic metal ion-catalyzed decarboxylation of β-keto dicarboxylic acids such as oxaloacetate.[284,285] Oxaloacetate is a substrate for malic enzyme which also requires a divalent cation.[252,255] Interestingly, PGDH does not require the asisstance of a divalent cation in order to exert the catalytic activity. The fact that PGDH has the substrate-specificity on β-keto monocarboxylic acid, 6-phosphogluconate, instead of δ-keto dicarboxylic acid, seems to be related with its different behavior towards the divalent metal cation from the other two enzymes.

1.4.2 Molecular Structure and Mechanism for Catalysis

Since the carbonyl group in a β-keto acid is an efficient electron sink, this acid is far more feasible to undergo decarboxylation than the corresponding β-hydroxy acid. Therefore, it could reasonably be postulated that dehydrogenation from a β-hydroxy acid occurs prior to decarboxylation. The hypothesis is consistent with the fact that oxalosuccinate, the putative intermediate, added to a solution of the enzyme is decarboxylated to α-ketoglutarate regardless the presence or absence of NADPH, but it is also reduced to isocitrate when NADPH is present.[249] Although these two activities are inherent properties of ICDH,[249,250] oxalosuccinate is not a free intermediate of the normal overall reaction; during the course of reaction with either ^{14}C-labeled isocitrate and $NADP^+$ or $^{14}CO_2$ cold α-ketoglutarate and NADPH, almost no radioactivity was incorporated into the oxalosuccinate.[251] These results can be explained either by the tight binding and a short life (consequently a low steady-state concentration) of the intermediate or by a concerted mechanism for dehydrogenation and decarboxylation. At any rate, the decarboxylation first leads to the formation of an enzyme-bound enol, which is subsequently protonated to form the ketoic product, 2-oxoglutarate as shown in Eq. 6.

$$\begin{array}{lcl} H_2CCOO^- & & H_2CCOO^- \\ HCCOO^- & \rightleftharpoons & HC \\ O{=}CCOO^- & CO_2 & {}^-O{-}CCOO^- \end{array} \quad \begin{array}{c} \\ H^+ \\ \rightleftharpoons \end{array} \quad \begin{array}{l} H_2CCOO^- \\ HCH \\ O{=}CCOO^- \end{array} \qquad (6)$$

Informations on the structure are mostly confined to those from studies with

chemical modification. In particular, glutamyl, (or/and aspartyl), cysteinyl, and lysyl residues are located at or near the substrate binding site, and thus have been implicated in the catalytic action of ICDH. Carboxyl group in the glutamyl residue might be a candidate for the acid-base catalyst in the dehydrogenation step, as suggested in the ME reaction.[256,286,287] A cysteinyl residue might be involved in the keto-enol tautomerization step as an acid-base catalyst. This residue locates near the C_3 of the substrate.[288–292] Lysyl residue(s) may participate in the substrate binding.[293–295]

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